(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 295 926 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
21.03.2018 Bulletin 2018/12

(51) Int Cl.:
A61K 8/73 *(2006.01)*

(21) Application number: **16382436.0**

(22) Date of filing: **19.09.2016**

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
MA MD

(71) Applicant: **Institut Univ. de Ciència i Tecnologia, S.A.**
08100 Mollet del Vallès Barcelona (ES)

(72) Inventors:
- **DERONCELÉ THOMAS, Víctor Manuel**
  08100 MOLLET DEL VALLÈS (ES)
- **MONTILLA AREVALO, Rafael**
  08100 MOLLET DEL VALLÈS (ES)
- **CASTELLS BOLIART, Josep**
  08100 MOLLET DEL VALLES (ES)
- **GARCÍA DE LA MARINA, Adrián**
  08100 MOLLET DEL VALLES (ES)

(74) Representative: **Oficina Ponti, SLP**
C. de Consell de Cent 322
08007 Barcelona (ES)

(54) **EXOPOLYSACCHARIDE-PROTEIN COMPLEX, A METHOD OF PREPARING SAID COMPLEX AND USES THEREOF**

(57) The present invention relates to an exopolysaccharide-protein complex obtained from a bacterium comprising: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins. The present invention further relates to a method for preparing said exopolysaccharide-protein complex and uses thereof.

EP 3 295 926 A1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to the field of biotechnology. In particular, the present invention relates to an exopolysaccharide-protein complex obtained from bacteria. The present invention further relates to a method of preparing said complex, a composition comprising thereof and uses thereof.

BACKGROUND OF THE INVENTION

[0002] Microbial exopolysaccharides (EPS) are synthesized by a wide variety of bacteria in natural ecological environments, mainly involved in the prevention of desiccation, protection against toxic and/or environmental stresses and adherence to surfaces (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001)(Nwodo, Green, & Okoh, 2012).

[0003] The number of EPSs produced by microbial fermentation has gradually increased with many applications in the food and pharmaceutical industries, among others, as their physiological activities differ from those of natural gums and synthetic polymers (Freitas, Alves, & Reis, 2011).

[0004] EPS production is among the biochemical strategies used by microorganisms present in hypersaline environments, in order to survive in high saline conditions. Among them, the genus *Halomonas* has received increasing interest as several species are able to produce significant quantities of EPS with high surface activity and/or have rheological properties and applications in food, cosmetic and pharmaceutical sectors (Poli, Anzelmo, & Nicolaus, 2010).

[0005] Microbial EPS are biopolymers with a high molecular weight having an extreme diversity in terms of chemical structure and composition. Polysaccharides are the most abundant component of the EPS but previous electron microscopy studies (Nevot, Deroncele, Lopez-Iglesias, et al., 2006; Nevot, Deroncele, Messner, Guinea, & Mercade, 2006), heavily emphasized that other macromolecules such as proteins can also be present.

[0006] Along with the general properties such as bio-compatibility, bio-degradability, renewability, flexibility, and eco-friendliness, EPS also offer some important biomedical properties, including antitumor activities (Bazani Cabral de Melo et al., 2015; Ye et al., 2016), antimutagenicity (Miranda et al., 2008), anti-ulcer (Rasulov et al., 1993), anti-inflammatory properties and immune-modulating activities (Ciszek-Lenda, Nowak, Srottek, Gamian, & Marcinkiewicz, 2011).

[0007] Anti-inflammatory and immune-modulating activities of EPS are drawing much attention and in lactic acid bacteria were related to the physicochemical properties and structural characteristics of their EPS (Gorska et al., 2014; Shao et al., 2014; Yasuda, Serata, & Sako, 2009). An important mechanism involved in the immunostimulatory activity of polysaccharides is their ability to enhance macrophage function (Beutler, 2004). Polysaccharides were reported to be the active immunomodulators that potentiate both innate and adaptive immunity. They can bind to pattern recognition receptors on the surface of macrophages, such as toll-like receptors, neutrophils, monocytes, NK cells and dendritic cells, and then trigger several signaling pathways to activate macrophages (Kim, Hong, Kim, & Han, 2011). NF-κB, a transcription factor that promote the expression of variety of molecules involved in immune, inflammatory and acute phase responses, including NO and tissue necrosis factor alpha (Li et al., 2015), plays an important role in this activation process.

[0008] Recently, a number of studies on the action mechanisms of polysaccharides have demonstrated that polysaccharides could also inhibit the tumor growth *in vivo* for their immunomodulatory activities (Sun, Li, Qi, Gao, & Lin, 2014; J. Yang, Li, Xue, Wang, & Liu, 2014; Zheng, Wang, & Li, 2015). They exert anti-tumor activity by boosting host's natural immune defense. Other work has suggested that chemopreventive activity of polysaccharides is based on their tumor anti-initiating activity through their modulation of carcinogen metabolism, in addition to the tumor anti-promoting activity through their anti-inflammatory activity (Gamal-Eldeen, Ahmed, & Abo-Zeid, 2009; Raafat, Gamal-Eldeen, El-Hussieny, Ahmed, & Eissa, 2014).

[0009] Document WO2015063240A1 describes the cosmetic and/or dermopharmaceutical use of an EPS produced by *Halomonas anticariensis,* specifically, for the treatment and/or care of the skin, and in particular, its use for inflammation, lipolysis, lipid accumulation and skin firmness. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-proteins and its use.

[0010] Document WO 2015/117985 relates to a strain of *Pseudomonas,* a cold-adapted bacteria, useful for cosmetic compositions. However, this document does not encompass the use of the crude exopolymeric material, including the associated protein. The composition discloses the use of a partial or completely hydrolized EPS derived from *Pseudomonas* for several purposes, including dermoprotection.

[0011] Document WO 2010/023178 A1 describes the use of bacterial polysaccharide derived from species of the genera *Bifidobacterium, Streptococcus* and *Lactobacillus,* some of which are considered as probiotic bacteria for treating inflammatory diseases, specifically, colitis or Crohn's disease. The application evaluated the anti-inflammatory potential of the purified EPS in a murine dendritic cell assay and/or an assay involving a human intestinal epithelial cell line. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-

proteins and its use.

[0012] US 7348420 B2 describes the recombinant expression of cell wall, cell surface, and secreted proteins of *Lactobacillus acidophilus* potentially useful for the treatment or prevention of cancer, particularly colon cancer. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-proteins and its use.

[0013] US 8129518 B2 provided synthetic polysaccharide antigens with anti-inflammatory or inflammatory immunomodulatory properties. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-proteins and its use. In this case the synthetic polysaccharide is composed mainly of lipopolysaccharide, and bacterial cell wall glycopeptides, also known as murein or peptidoglycan, from both Gram negative and Gram positive bacteria.

[0014] US 8088605 B2 discloses a delivery system for active molecule comprising exopolysaccharide micelles produced by a *Lactobacillus* strain. These includes active molecules such as DNA, RNA, protein, peptide, peptidomimetic, virus, bacteria, nutraceutical product and pharmaceutical agent with analgesic, anesthetic, antibiotic, anticancer, anti-inflammatory, and antiviral properties. However, these pharmaceutical agents are not produced by the bacterium.

[0015] WO 2009/127057 A1 discloses a skin care composition comprising one exopolysaccharide derived from a microbial mat. This document discusses the use of different compositions for cosmetic or therapeutic approaches and provides examples for evaluating the effects of the EPSs on the synthesis of hyaluronic acid, lipid synthesis, among others, related with cosmeceuticals applications. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-proteins and its use as anti-inflammatory or immunomodulatory agents.

[0016] CN 104694594 discloses a preparation method of a sea cucumber epiphytic *Bacillus subtilis* exopolysaccharide that can be used in the anti-tumor medicines, cosmetic additives and other fields. The principal application exposed in this document is related with the application of this EPS in bacterial and plant pathogen growth inhibition, a peroxide scavenger, anti-tumor and inhibition of microapplication.

[0017] US9095733 discloses methods of using polysaccharides for applications in topical personal care products, cosmetics, and for wrinkle reduction compositions. Particularly, this document provides compositions of microalgal cells with high value cosmeceutical ingredients such as carotenoids, polyunsaturated fatty acids, moisturizing polysaccharides, superoxide dismutase, and other components. However, this document does not disclose the combination of extracellular polysaccharides and selected associated-proteins and its use as anti-inflammatory or immunomodulatory agents.

[0018] WO 2013/082915 A1 discloses a strain of exopolysaccharide-secreting *Lactobacillus brevis,* and its applications in medicaments, healthcare products and food products for immunity enhancement. The exopolysaccharide extract is obtained after precipitating protein via trichloroacetic acid method to remove the protein.

[0019] Thus, the above prior art known processes relating to the production of EPS from microbial sources do not disclose the combined effect of polysaccharide fraction with selected extracellular EPS-associated proteins.

[0020] The present inventors have surprisingly found that an exopolysaccharide-protein complex secreted by bacteria exhibits unique immunomodulating properties, is non-cytotoxic and non-proliferating to normal cell lines. The exopolysaccharide-protein complex ameliorates pro-inflammatory chemokines expression and induces the production of other anti-inflammatory cytokines of cells in culture. It has the activity of inhibiting growth of tumor cell lines. Accordingly, the complex can be used for treatment or prevention of diseases in which inflammation and immunomodulation are critical or as adjuvant in medical treatment, such as those related to an imbalance of the production of anti-inflammatory or proinflammatory cytokines, and also for preparing anti-tumor drugs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021]

Fig. 1 shows sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel of EPCx. Analysis from SDS-PAGE gel indicates that the two principal bands have molecular weights of -33 kDa and -55 kDa, respectively.

Fig. 2 shows the effect of EPCx on NFκB activation in LPS-stimulated keratinocytes.

Fig. 3 shows the effect of EPCx on NFκB activation in LPS-stimulated THP-1 cells.

Fig. 4 shows the growth inhibition of EPCx at different concentrations against cancer cell lines *in vitro.*

Fig. 5 shows the partial 16S rRNA gene sequence of the isolate according to example 1.

SUMMARY OF THE INVENTION

[0022] A first object of the invention relates to an exopolysaccharide-protein complex obtained from a bacterium comprising: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins.

[0023] A second object of the invention relates to a method of preparing an exopolysaccharide-protein complex according to the first object.

[0024] A third object of the invention relates to a composition (pharmaceutical, nutraceutical or cosmeceutical) comprising an exopolysaccharide-protein complex according to the first object.

[0025] A fourth object of the invention relates to the use of the composition according to the third object of the invention and the exopolysaccharide-protein complex according to the first object of the invention.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0026] In order to facilitate the comprehension of this invention, the meanings of some terms and expressions as used in the context of the invention are included.

[0027] As used herein, the term "isolated" should be considered to mean material removed from its original environment in which it naturally occurs, for example, a bacterial strain from hypersaline environment.

[0028] As used herein, the term "EPS" or "exopolysaccharide" or "EPSx" should be understood to mean high molecular weight polymers that are composed of sugar residues and expressed by bacteria.

[0029] As used herein, "activation of NF-κB (nuclear factor-kappa B)" means the process by which stimulation of NF-κB mediated by Toll-like receptors activates NF-κB, subsequently facilitating increased transcription of mRNA coding for intracellular production of particular chemokines and cytokines and subsequent translation of the transcribed mRNA, resulting in increased amounts of particular cytokines and chemokines that are both present intracellularly and released by the eukaryotic cell into the intercellular environment.

[0030] As used herein, "interleukin" means any of a group of cytokines (secreted signaling molecules) that were first seen to be expressed by white blood cells. Interleukins are commonly designated using an abbreviation: e.g. IL-6, IL-8, etc.

[0031] As used herein, the term "immunomodulatory" refers to its ability to modulate the response of cells of the human immune system.

[0032] As used herein, "anti-inflammatory" refers to the ability to induce the production of interleukin-10, a potent anti-inflammatory cytokine and to block the production of interleukin 12, a cytokine pro-inflammatory nature.

[0033] As used herein, "cancer" and "tumor" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth.

[0034] As used herein, the term "anticancer activity" as used herein refers to preferential cytotoxic effect against tumor cells without any significant adverse effects to normal cells under the same conditions of exposure.

[0035] As used herein, the term "nutraceutical" refers to any compounds or chemicals that can provide dietary or health benefits when consumed by humans or animals.

[0036] As used herein, the term "cosmeceutical composition" refers to a composition that is employed as both a cosmetic composition and as a pharmaceutical composition.

[0037] As used herein, the term "adjuvant" refers to an embodiment of the invention provided to a subject in conjunction with a medical treatment plan.

[0038] As used herein, "immunomodulation", "immunomodulatory", and similar terms refer to the ability to modify the immune responses in a subject in a way that may have healthful benefits, such as to produce an anti-inflammatory or an immunostimulatory effect.

[0039] As used herein, "RT-PCR" means reverse transcription polymerase chain reaction (RT-PCR), a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. This can either be a 1 or 2 step process.

EMBODIMENTS

[0040] In a first aspect the present invention relates to an exopolysaccharide-protein complex (also called herein EPCx) obtained from a bacterium, preferably wherein said bacterium is from a genus selected from *Halomonas, Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, and Bacillus,* more preferably from genus *Halomonas,* still more preferably from *Halomonas elongata sp.,* comprising: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins. In this context, the term "comprising" should be understood as the components of the exopolysaccharide-protein complex are not limited to (i) a crude exopolysaccharide, and (ii)

exopolysaccharide-associated proteins.

[0041] In another embodiment, said exopolysaccharide-protein complex obtained from a bacterium consists of: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins. In this context, the term "consists of" should be understood as the components of the exopolysaccharide-protein complex are exclusively (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins.

[0042] In a preferred embodiment, the exopolysaccharide-protein complex of the present invention relates to an exopolysaccharide-protein complex comprising: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins, wherein the crude exopolysaccharide is a heteropolymer comprising or consisting of:

- glucose units;
- galactose units;
- uronic acid units; and
- other sugar units selected from rhamnose, glucosamine and a mixture of rhamnose and glucosamine.

[0043] In a further preferred embodiment, the exopolysaccharide-protein complex of the invention comprises or consists of:

(a) 30 to 60 wt% glucose;
(b) 30 to 50 wt% galactose;
(c) 5 to 10 wt % uronic acids; and
(d) 1 to 10wt% of other sugar units,

providing that the sum of the components of the crude exopolysaccharide is 100 wt%, i.e. the components (a), (b), (c) and (d) must amount 100 wt% if components (a), (b), (c) and (d) are the only components in the exopolysaccharide-protein complex or the components (a), (b), (c) and (d) plus any further component(s) must amount 100 wt% if components (a), (b), (c) and (d) are not the only components in the exopolysaccharide-protein complex.

[0044] In a further preferred embodiment, in the exopolysaccharide-protein complex, the crude exopolysaccharide further comprises sulfate, preferably at a concentration from 2 to 10 wt %.

[0045] In a further preferred embodiment, in the exopolysaccharide-protein complex, the exopolysaccharide-associated proteins include two bands of molecular weight as obtained by SDS-PAGE. In particular, the EPCx has a protein profile comprising at least, according to the SDS-PAGE technique, 12 detectable bands, including two principal bands, corresponding, respectively, to molecular weights (approximate molecular weights given in relation to molecular standards, notably provided by Bio-Rad Laboratories) ranging between:

- band 1: 30 kDa and 40 kDa, in particular 33kDa;
- band 2: 51 kDa and 60kDa, in particular 55kDa.

[0046] In a second aspect, the present invention relates to a method of preparing an exopolysaccharide-protein complex according to the first aspect of the invention, including each of the embodiments comprised in said first aspect and combinations thereof.

[0047] The method of preparing an exopolysaccharide-protein complex comprises the steps of:

- culturing one or more bacteria from genus including, but not limited thereto, Halomonas, *Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, Bacillus* or any other bacterium isolated from a hypersaline environment, the term "hypersaline" referred to a kind of extreme environments that have salt concentrations much greater than that of seawater, often close to or exceeding salt saturation;
- isolating the exopolysaccharide-protein complex from the secreted fraction of the culture.

[0048] In a preferred embodiment, the genus is selected from *Halomonas, Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, and Bacillus.* In a more preferred embodiment, said bacteria genus is *Halomonas* and in a more preferred embodiment, *Halomonas elongata sp.* is used as a bacterium belonging to *Halomonas* genus.

[0049] The medium suitable to cultivate the above mentioned bacteria includes a synthetic medium comprising a carbon source selected from the group consisting of: lactose, maltose, glucose, galactose, sucrose, glycerol and mixtures thereof. Preferably, the carbon source is selected from the group consisting of: glucose, lactose, sucrose, and mixtures thereof. In a more preferred embodiment, the carbon source is lactose.

[0050] In a particular embodiment, the other fermentation medium components are: potassium phosphate dibasic (K_2HPO_4), 0.5 -1.0 wt%; potassium phosphate monobasic (KH_2PO_4), 0.1-0.5wt %; sodium chloride (NaCl), 5.0-10.0 wt

%; magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.01-0.05 wt%; ammonium sulfate $(NH_4)_2SO_4$, 0.05 wt %-0.5wt % and peptone, 0.02-0.1 wt %.

[0051] In a preferred embodiment, before culturing bacteria, the suitable medium for culturing is a synthetic medium with a pH value ranging from 6 to 8. In a more preferred embodiment, the suitable medium has a pH value of 7.

[0052] In another preferred embodiment, the culture step is conducted in fermenters operating at a temperature ranging from 25°C to 37°C. In a more preferred embodiment, the temperature is 32°C.

[0053] In a particular embodiment, the present invention provides a fermentation process comprising a fermentation step allowing to grow a strain of the corresponding bacteria, preferably wherein said bacteria is from a genus selected from *Halomonas, Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, and Bacillus,* more preferably from genus *Halomonas,* still more preferably from *Halomonas elongata sp.,* in a suitable medium in a fermenter under conditions of agitation sufficient to maintain a homogenous culture and limited aeration such that dissolved oxygen pressure (pO2) within the culture is around 20 to 40 % for most of the fermentation step. Preferably, pO2 within the culture is 30 % in the fermentation step.

[0054] As mentioned above, the inventive process of preparing an exopolysaccharide-protein complex comprises the step of isolating the exopolysaccharide-protein complex from the secreted fraction of the culture. Said isolation can be carried by removing other molecules present in the culture media by alcohol precipitation. Non-limiting examples of alcohols which can be used include ethanol, isopropanol, and methanol. In particular, the isolation and purification of the EPCx after alcohol precipitation can be conducted by tangential flow filtration methods using ultrafiltration membranes. Preferably, said membranes have a MWCO of 30 kDa and the retentate recovered after the ultrafiltration comprises the exopolysaccharide and the EPS-associate proteins which can be recovered. The tangential flow filtration can act to both diafilter and concentrate the EPCx.

[0055] In a third aspect, the present invention relates to a composition (pharmaceutical, nutraceutical or cosmeceutical) comprising an exopolysaccharide-protein complex according to the first object of the invention, including each of the embodiments comprised in said first aspect and combinations thereof.

[0056] In a preferred embodiment, said composition further comprises a biological response modifier selected from the group consisting of lymphokine, interleukin, growth factor and NFkB factor.

[0057] In a fourth aspect, the present invention relates to a composition comprising an exopolysaccharide-protein complex according to the first object of the invention, including each of the embodiments comprised in said first aspect and combinations thereof, for use in stimulating an immune response in a subject for preventing or treating a disease selected from cancer or a disease associated to undesirable inflammatory activity.

[0058] In a preferred embodiment, said disease associated to undesirable inflammatory activity is selected from allergy, Alzheimer's disease, arthritis, autoimmune deficiency syndrome, celiac disease, diabetes mellitus, gastrointestinal disorder, inflammatory bowel disease, interstitial cystitis, skin disorders, acne, arteritis, arthritis, cancer, cellulitis, dermatitis and cardiovascular diseases.

[0059] In another preferred embodiment, said cancer is liver cancer or a hepatocellular carcinoma.

[0060] In another preferred embodiment, said exopolysaccharide-protein complex according to the first object of the invention, including each of the embodiments comprised in said first aspect and combinations thereof, is used as an anticancer agent or adjuvant agent for cancer therapies.

[0061] In another embodiment, the present invention provides an *in vitro* toxicity test to evaluate the cytotoxic potential of EPCx on non-tumor cell lines. The cytotoxicity evaluation of each concentration of EPCx was performed by using the MTT Cell Proliferation Assay in order to determine the concentrations which are not harmful to Human Epidermal Keratinocytes (HEK) and human monocyte-like cells (THP-1).

[0062] As shown in the example section, the EPCx show *in vitro* anti-proliferative activity against a panel of one or more cancer cell lines, including human lung adenocarcinoma cell line (H1975), Human melanoma cell line (A375), and human hepatocellular carcinoma cell line (HepG2).The anticancer properties of EPCx was ascertained by MTT assay and showed that EPCx significantly inhibit the growth of human lung adenocarcinoma cell line (H1975), Human melanoma cell line (A375), and human hepatocellular carcinoma cell line (HepG2).

[0063] The immunomodulatory and anti-proliferative properties of EPCx were ascertained by the analysis of the induced activation of human primary keratinocytes (HEK) and human monocyte-like cells (THP-1) stressed with LPS (lipopolysaccharide), analyzing the expression of mRNA for the cytokines IL-6, IL-8, IL-10 and TNF-α by real-time PCR and the NF-κB activation using a luciferase reporter gene assay.

[0064] The immunomodulatory and anti-cancer properties of the EPCx, are showed preferably at EPCx concentrations between 0.01 mg/ml to 1.0 mg/ml.

[0065] Accordingly, the inventors have surprisingly and unexpectedly found that an EPCx obtained from a bacterium, preferably wherein said bacterium is from a genus selected from *Halomonas, Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, and Bacillus,* more preferably from genus *Halomonas,* still more preferably from *Halomonas elongata sp.,* in accordance with the invention, can be used as anti-inflammatory and/or inmunomodulator agent, and is in particular able to inhibit a pro-inflammatory stimulation of normal cells in culture. In

addition EPCx exhibits tumor anti-promoting properties in cancer cell lines culture.

[0066] The present invention will be more clearly understood with the help of the following examples, without being the present invention limited thereto and included only for illustrative purposes only, showing isolation and characterization of bacteria, the preparation and characterization of EPCx and assays for biological activities in accordance with the invention.

EXAMPLES

Example 1: Isolation and Identification of the microorganism.

Isolation of the Microorganism.

[0067] A microorganism included in the present invention is isolated from a rock salt from solar salternsin Cardona(Spain) by serial dilution and plating on MH agar medium(Ventosa, Garcia, Kamekura, Onishi, & Ruizberraquero, 1989). The plates were incubated at 32°C for 3 to 5 days, and bacterial colonies were isolated in pure form and maintained on slopes of the same medium.

[0068] To evaluate the EPS production capability, the bacterium isolates was grown in MY medium (Moraine & Rogovin, 1966) supplemented with 5-10 % NaCl for 3-5 days at 32°C under continuous shaking (120 rpm). The EPS from the growing culture was isolated using the method as described by Quesada et al. (Quesada, Bejar, & Calvo, 1993) and used for quantification and chemical analysis.

Identification of the microorganism

[0069] The selected microorganism was analyzed by physiological and biochemical methods following standard microbiological methods (Table 1). As a result, based on physiological and biochemical analysis thereof, the microorganism of the present invention was confirmed to have similarity to *Halomonas* genus.

Table 1. Morphological and physiological characteristics of the isolated microorganism.

Character	
Morphological characters	
Colony morphology	Cream, circular
Gram nature	-
Cell shape	Rod
Cell arrangement	Single
Motility	+
Pigmentation	-
Physiological characters	-
pH range for growth	5-11
pH optimum for growth	8
Temp. range for growth (°C)	22-40
Temp. optimum for growth (°C	32
NaCl range for growth (%)	1-20
NaCl optimum for growth (%)	5
Growth on King's B medium	+
Growth on McConky agar	-
Biochemical characters	
Voges-Proskauer test	-
Citrate utilization	+

(continued)

Biochemical characters	
Methyl red test	-
Production of	
Gelatinase	-
Urease	-
Catalase	+
Nitrate reductase	+
H2S	-
Lysine decarboxylase	-
Arginine decarboxylase	-
Ornithine decarboxylase	-
Indole	5
Phenylalanine deaminase	+
Tryptophan deaminase	-
Tentative identity	*Halomonas*

[0070] In order to identify more precisely, the present inventors analyzed base sequence of ribosome small subunit gene. Particularly, genomic DNA was separated by using MasterPure TM DNA purification from Epicentre ® Biotechnologies Germany according to the manufacturer's instruction. PCR mediated amplification of 16S rDNA and purification of the PCR product was carried out as described previously (Rainey, WardRainey, Kroppenstedt, & Stackebrandt, 1996). As a result of database analysis on the decided base sequence using NCBI (National Center for Biological Information) BLAST, the microorganism was confirmed to have similarity to *Halomonas* genera. However, it had a little difference from the typical *Halomonas elongata,* so that the microorganism of the present invention was finally named as *Halomonas elongata.*

[0071] Partial sequencing of the 16S rRNA gene (844 bp) indicated that the strain belongs to the species *Halomonas elongata.* The partial 16S rRNA gene sequence is shown in figure 5.

Example 2. Preparation and Isolation of the Exopolysaccharide-Protein Complex (EPCx) excreted by *Halomonas elongata* according to example 1.

a) *Method of Culturing of Strain the Species Halomonas elongata*

[0072] The strain of the species *Halomonas elongata* was cultured in a fermenter, at 32°C and at a pH of 7.5, whose broth contained (g L^{-1}): 100 NaCl; 50 Lactose; 7 K_2HPO4; 2 KH_2PO4; 0.1 $MgSO4 \cdot 7H_2O$; 1 $(NH_4)_2SO_4$ and 0.5 Peptone. An inoculum was prepared with 10% (v/v) of a pre-culture and the duration of the fermentation was extended to 72 hours. The reactors are operated in batch mode, and dissolved oxygen was controlled by the agitation (300 to 900 rpm) at $pO_2 \approx 30\%$.

b) *Purification of EPCx*

[0073] The bacteria were separated from the broth by centrifugation at 12,000 g for 45 min. The resulting clear solution was subjected to ultrafiltration and dialysis using an installation for ultrafiltration (Sartocon® Slice Cassette, Sartorius Stedim), membrane exclusion limit 30 KDa. If necessary, the final solution may be lyophilized and purified obtaining an exopolysaccharide-protein complex with a yield of 60-80%.

Example 3. Physical-Chemical Characterization of EPCx Produced by *Halomonas elongata*

[0074] The content of neutral and acid monosaccharides of the exopolysaccharide obtained according to Example 2 was determined by a method described by Honda et al.(Honda et al., 1989) and Yang et al. (X. B. Yang, Zhao, Wang,

Wang, & Mei, 2005; X. B. Yang, Zhao, Zhou, et al., 2005). Briefly, the purified polysaccharide sample (1 mg) was hydrolyzed with 1 ml of 2 M trifluoroacetic acid at 120°C for 2 h, derivatized with 1-phenyl-3-methyl-5-pyrazolone, and subsequently analyzed by high-performance liquid chromatography with detection by absorbance monitoring at 245 nm.

[0075] The percent relationship of sugars obtained was 30-60% of glucose, 30-50% galactose, 5-10% of glucuronic acid, 1-10% of rhamnose and 1-10% of glucosamine, being the amounts consistent with a total of 100%.

[0076] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)was carried out with a 4% stacking and a 9% separating gel. The EPCx sample was dissolved at 10 mg/mL in distilled water and added at 1:3 volume ratio into a buffer solution of 0.5% SDS with 1 %-mercaptoethanol, and then heated to boiling for 5 min. The gels were stained with Coomassie Brilliant Blue R-250 to visualize proteins. 12 detectable bands, including three principal bands of 38 kDa, 46 kDa and 54 kDa were observed (see figure 1).

Example 4. Effect of exopolysaccharide-protein complex on non-tumor cell lines (Viability in Vitro).

[0077] The cytotoxicity evaluation of exopolysaccharide-protein complex was performed by using the MTT assay in order to determine the concentrations which are not harmful to Human Epidermal Keratinocytes (HEK) and human monocyte-like cells (THP-1). Proliferation of cells lines was measured based on the mitochondria-dependent reduction of yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), to generate reducing equivalents such as NADH and NADPH. Briefly, 5×10^3 cells in 100 μL per well were plated in 96-well tissue culture plates for 24 h. Cells were incubated for 72 h in the presence of different concentrations of EPS (1, 0.1 and 0.01 mg/ml). After that, 20 μL of MTT solution (5 mg/mL in PBS) were added into each well and followed by further incubation for 4-5 h. The resulting intracellular purple formazan can be solubilised and quantified by spectrophotometric means. The results are given in Table 2 below.

Table 2. Effect of exopolysaccharide-protein complex non-tumor cell lines.

Cell line	Concentration (mg/ml)	Cell viability % of MTT conversion
HEK	1 0.1 0.01	101 98 102
THP-1	1 0.1 0.01	102 97 104

[0078] No viability alteration and no significant changes in cells proliferation were observed at the exopolysaccharide-protein complex concentrations tested in this assay on normal cell lines (table 2).

Example 5. Effects of exopolysaccharide-protein complex in HEK and THP-1 Cells Stressed with LPS.

[0079] The capacity of EPS to modulate the inflammatory response was evaluated *in vitro* in human primary keratinocytes (HEK) and human monocyte-like cells (THP-1) stressed with LPS (lipopolysaccharide). The cells were pretreated with different concentrations of exopolysaccharide-protein complex (1, 0.1 and 0.01 mg/ml) for 24 h and with 10 μg/ml of LPS for 24h. Controls of HEK and THP-1 cells treated only with the different concentrations of exopolysaccharide-protein complex (1.0, 0.1 and 0.01 mg/ml) were also prepared.

[0080] Expression of mRNA for the cytokines IL-6, IL-8, IL-10 and TNF-α was analyzed by real-time PCR and the NF-κB activation was measured using a luciferase reporter gene assay.

[0081] *Real-time PCR:* Total RNA was extracted with the RNA Isolation Kit (ThermoFischer Scientific) according to the manufacturer's recommendations. 1 ng of RNA was reverse transcribed into complementary DNA (cDNA) using Superscript One-Step RT-PCR kit with platinum Taq according to the instructions (Invitrogen). Quantification rests on the measure of threshold cycles (CT), which are measured at the beginning of the exponential phase of the reaction and on the normalization of the internal standard curve obtained with the reference gene.

[0082] When HEK and THP-1 cells were pretreated with exopolysaccharide-protein complex (EPCx) before stimulation with LPS, we did not observe the upregulation of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α), as occurred in the LPS-treated cells. However, cells pretreated with EPCx before stimulation with LPS shows a downregulation of anti-inflammatory cytokine, IL-10 (tables 3 and 4).

Table 3. Gene expression (mRNA, real-time PCR) of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) and anti-inflammatory cytokine (IL-10) in keratinocytes stresses with LPS and treated with different concentrations of EPCx (1.0, 0.1 and 0.01 mg/ml).

Gene expression (mRNA, real-time PCR)				
	IL-6	IL-8	TNF-α	IL-10
Control	3	2	4	1
LPS	62	59	67	7
EPCx 0.01	12	17	21	11
EPCx 0.1	17	14	27	18
EPCx 1.0	15	18	31	22

Table 4. Gene expression (mRNA, real-time PCR) of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) and anti-inflammatory cytokine (IL-10) in human monocyte-like cells stresses with LPS and treated with different concentrations of EPCx (1, 0.1 and 0.01 mg/ml).

Gene expression (mRNA, real-time PCR)				
	IL-6	IL-8	TNF-α	IL-10
Control	1.5	3	2	1
LPS	73	82	67	5
EPCx 0.01	21	27	32	12
EPCx 0.1	27	30	44	12
EPCx 1.0	15	18	31	15

[0083] *Measurement of NF-κB activation*: NF-κB activation was measured using a luciferase reporter gene assay. For this assay THP-1 macrophages (1×10^5 cells·well^{-1}) were transfected with the pNF-κBluciferase reporter gene construct (Stratagene) using Lipofectamine LTX plus (Invitrogen). Sixteen hours after transfection, different concentrations of EPS (1,0.1 and 0.01 mg/ml) were added and the incubation was continued for a further 8 h. The cells were then washed with NaCl/P_i and lysed using lysis buffer (200 μL·well^{-1}) (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol and 1% Triton X-100). Lysed cells were centrifuged (5 min, 9000 g) and stored at -80 °C until assay. Luciferase activity was measured using luciferin (1 mM in glycylglycine buffer, 300 μL·sample^{-1}) in a luminometer at 562 nm.

[0084] EPCx dose-dependently downregulated the NF-κB transcription activity in keratinocyte cells (Figure 2), whereas in EPCx-treated human monocyte-like cells, the NF-kB levels were comparable to those obtained in the THP-1 cells treated with LPS (Figure 3).

In vitro anti-tumor activities

[0085] The MTT assay was used for measuring the proliferation of the tumor cells. Briefly, human hepatocellular carcinoma cell line (HepG2), human breast adenocarcinoma cell line (MCF7), human lung adenocarcinoma cell line (H1975) and Human melanoma cell line (A375) were seeded at a density of 4×10^4 cells/mL in a volume of 0.1 mL in 96-well plates, respectively. After 24 h, different concentrations of EPCx (1.0, 0.1 and 0.01 mg/ml) were dissolved in the medium was added to each well and incubated for 48 h at 37 °C in a CO_2 incubator. 5-Fu (5-fluorouracil) was used as the positive control. After the incubation, 20 μL of MTT solution (5 mg/mL) were added into each well and followed by further incubation for 4-5 h. The culture media were then removed and 100 μL of DMSO was added to each well for 1 h. Absorbance at 570 nm was detected by microplate ELISA reader. The inhibition ratio of the tumor cells proliferation was determined, the results are shown in figure 4.

[0086] MTT assay showed that EPCx markedly inhibited proliferation of human hepatocellular carcinoma cell line (HepG2) in a dose-dependent manner (Figure 4), with little effect on growth of human breast adenocarcinoma cell line (MCF7), human lung adenocarcinoma cell line (H1975) and Human melanoma cell line (A375). The potency of EPCx (at 1.0 mg/mL) to HepG2 cells was found to be similar to 5-fluorouracil (5-FU, IC50 was 10.0 μmol/L).

[0087] The foregoing description of preferred embodiments and examples is intended only to acquaint others skilled in the art with Applicants' invention, its principles, and its practical application so that others skilled in the art may adapt and apply the invention in its numerous forms, as they may be best suited to the requirements of a particular use. This

detailed description and its specific examples, while indicating preferred embodiments of this invention, are intended for purposes of illustration only. This invention, therefore, is not limited to the preferred embodiments described in this specification, and may be variously modified.

REFERENCES

[0088]

Bazani Cabral de Melo, F. C., Borsato, D., de Macedo Junior, F. C., Mantovani, M. S., Luiz, R. C., & Pedrine Colabone Celligoi, M. A. (2015). Study of levan productivity from Bacillus subtilis Natto by surface response methodology and its antitumor activity against HepG2 cells using metabolomic approach. Pakistan Journal of Pharmaceutical Sciences, 28(6), 1917-1926.

Beutler, B. (2004). Innate immunity: an overview. Molecular Immunology, 40(12), 845-859. Ciszek-Lenda, M., Nowak, B., Srottek, M., Gamian, A., & Marcinkiewicz, J. (2011). Immunoregulatory potential of exopolysaccharide from Lactobacillus rhamnosus KL37. Effects on the production of inflammatory mediators by mouse macrophages. International Journal of Experimental Pathology, 92(6), 382-391.

De Vuyst, L., De Vin, F., Vaningelgem, F., & Degeest, B. (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. International Dairy Journal, 11(9), 687-707.

Freitas, F., Alves, V. D., & Reis, M. A. M. (2011). Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends in Biotechnology, 29(8), 388-398. Gamal-Eldeen, A. M., Ahmed, E. F., & Abo-Zeid, M. A. (2009). In vitro cancer chemopreventive properties of polysaccharide extract from the brown alga, Sargąssum latifolium. Food and Chemical Toxicology, 47(6), 1378-1384.

GarcIA Sanz, M. N., Ferrer Montiel Antonio, V., Soley Astals, A., & AlmiÑAna DomÉNech, N. (2015). WO Patent No. WO 2015/063240 A1.

Gorska, S., Schwarzer, M., Jachymek, W., Srutkova, D., Brzozowska, E., Kozakova, H., et al. (2014). Distinct Immunomodulation of Bone Marrow-Derived Dendritic Cell Responses to Lactobacillus plantarum WCFS1 by Two Different Polysaccharides Isolated from Lactobacillus rhamnosus LOCK 0900. Applied and Environmental Microbiology, 80(20), 6506-6516.

Honda, S., Akao, E., Suzuki, S., Okuda, M., Kakehi, K., & Nakamura, J. (1989). HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHY OF REDUCING CARBOHYDRATES AS STRONGLY ULTRAVIOLET-ABSORBING AND ELECTROCHEMICALLY SENSITIVE 1-PHENYL-3-METHYL-5-PYRAZOLONE DERIVATIVES. Analytical Biochemistry, 180(2), 351-357.

Kim, H. S., Hong, J. T., Kim, Y., & Han, S.-B. (2011). Stimulatory Effect of beta-glucans on Immune Cells. Immune network, 11(4), 191-195.

Laemmli, U. K. (1970). CLEAVAGE OF STRUCTURAL PROTEINS DURING ASSEMBLY OF HEAD OF BACTERIOPHAGE-T4. Nature, 227(5259), 680-&.

Li, J., Qian, W., Xu, Y., Chen, G., Wang, G., Nie, S., et al. (2015). Activation of RAW 264.7 cells by a polysaccharide isolated from Antarctic bacterium Pseudoaltermonas sp S-5. Carbohydrate Polymers, 130, 97-103.

Loing, E., Briatte, S., Vayssier, C., Beaulieu, M., & Dionne, P. (2009). WO Patent No. WO 2009/127057 A1.

MercadÉ Gil Ma, E., CamON Fonseca, O., & Montes LOPez Ma, J. (2015). WO Patent No. WO 2015/117985 A1.

Miranda, C. C. B. O., Dekker, R. F. H., Serpeloni, J. A., Fonseca, E. A. L., Couls, L. M. S., & Barbosa, A. M. (2008). Anticlastogenic activity exhibited by botryosphaeran, a new exopolysaccharide produced by Botryosphaeria rhodina MAMB-05. International Journal of Biological Macromolecules, 42(2), 172-177.

Moraine, R. A., & Rogovin, P. (1966). KINETICS OF POLYSACCHARIDE B-1459 FERMENTATION. Biotechnology and Bioengineering, 8(4), 511-&.

Nevot, M., Deroncele, V., Lopez-Iglesias, C., Bozal, N., Guinea, J., & Mercade, E. (2006). Ultrastructural analysis of the extracellular matter secreted by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3. Microbial Ecology, 51(4), 501-507.

Nevot, M., Deroncele, V., Messner, P., Guinea, J., & Mercade, E. (2006). Characterization of outer membrane vesicles released by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3. Environmental Microbiology, 8(9), 1523-1533.

Nwodo, U. U., Green, E., & Okoh, A. I. (2012). Bacterial Exopolysaccharides: Functionality and Prospects. International Journal of Molecular Sciences, 13(11), 14002-14015.

Poli, A., Anzelmo, G., & Nicolaus, B. (2010). Bacterial Exopolysaccharides from Extreme Marine Habitats: Production, Characterization and Biological Activities. Marine Drugs, 8(6), 1779-1802.

Quesada, E., Bejar, V., & Calvo, C. (1993). EXOPOLYSACCHARIDE PRODUCTION BY VOLCANIELLA EURIHALINA. Experientia, 49(12), 1037-1041.

Raafat, E. M., Gamal-Eldeen, A. M., El-Hussieny, E. A., Ahmed, E. F., & Eissa, A. A. (2014). Polysaccharide extracts

of the brown alga Sargassum asperifolium possess in vitro cancer chemopreventive properties. Natural Product Research, 28(24), 2304-2311.
Rainey, F. A., WardRainey, N., Kroppenstedt, R. M., & Stackebrandt, E. (1996). The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage: Proposal of Nocardiopsaceae fam nov. International Journal of Systematic Bacteriology, 46(4), 1088-1092.
Rasulov, M. M., Kuznetsov, I. G., Slutskii, L. I., Velikaia, M. V., Zabozlaev, A. G., & Voronkov, M. G. (1993). The ulcerostatic effect of the exopolysaccharide from Bacillus mucilaginosus and its possible mechanisms. Biulleten' eksperimental'noi biologii i meditsiny, 116(11), 504-505.
Shao, L., Wu, Z., Zhang, H., Chen, W., Ai, L., & Guo, B. (2014). Partial characterization and immunostimulatory activity of exopolysaccharides from Lactobacillus rhamnosus KF5. Carbohydrate Polymers, 107, 51-56.
Sun, Z., Li, M. J., Qi, Q. S., Gao, C. J., & Lin, C. S. K. (2014). Mixed Food Waste as Renewable Feedstock in Succinic Acid Fermentation. Applied Biochemistry and Biotechnology, 174(5), 1822-1833.
Ventosa, A., Garcia, M. T., Kamekura, M., Onishi, H., & Ruizberraquero, F. (1989). BACILLUS-HALOPHILUS SP-NOV, A MODERATELY HALOPHILIC BACILLUS SPECIES. Systematic and Applied Microbiology, 12(2), 162-166.
Yang, J., Li, X., Xue, Y., Wang, N., & Liu, W. (2014). Anti-hepatoma activity and mechanism of corn silk polysaccharides in H22 tumor-bearing mice. International Journal of Biological Macromolecules, 64, 276-280.
Yang, X. B., Zhao, Y., Wang, Q. W., Wang, H. F., & Mei, Q. B. (2005). Analysis of the monosaccharide components in Angelica polysaccharides by high performance liquid chromatography. Analytical Sciences, 21(10), 1177-1180.
Yang, X. B., Zhao, Y., Zhou, S. Y., Liu, L., Wang, H. F., & Mei, Q. B. (2005). Analysis of monosaccharide composition in Angelica polysaccharides by precolumn derivatization high performance liquid chromatography. Chinese Journal ofAnalytical Chemistry, 33(9), 1287-1290.
Yasuda, E., Serata, M., & Sako, T. (2009). Suppressive Effect on Activation of Macrophages by Lactobacillus casei Strain Shirota Genes Determining the Synthesis of Cell Wall-Associated Polysaccharides (vol 74, pg 4746, 2008). Applied and Environmental Microbiology, 75(4), 1221-1221.
Ye, S., Zhang, J., Liu, Z., Zhang, Y., Li, J., & Li, Y. O. (2016). Biosynthesis of selenium rich exopolysaccharide (Se-EPS) by Pseudomonas PT-8 and characterization of its antioxidant activities. Carbohydrate Polymers, 142, 230-239.
Zhang, F., Liu, Y. E., Wang, W., Zhao, Y. U., & Li, Z. (2015). CN Patent No. CN 104694594 A.
Zheng, Y., Wang, W.-d., & Li, Y. (2015). Antitumor and immunomodulatory activity of polysaccharide isolated from Trametes orientalis. Carbohydrate Polymers, 131, 248-254.

Claims

1. An exopolysaccharide-protein complex obtained from a bacterium comprising: (i) a crude exopolysaccharide, and (ii) exopolysaccharide-associated proteins.

2. The exopolysaccharide-protein complex according to claim 1, wherein the crude exopolysaccharide is an heteropolymer comprising glucose, galactose, uronic acid units and other sugar units selected from rhamnose, glucosamine and a mixture of rhamnose and glucosamine.

3. The exopolysaccharide-protein complex according to claim 2, wherein the crude exopolysaccharide comprises:30 to 60 wt% glucose, 30 to 50 wt% galactose, 5 to 10 wt % uronic acids and 1 to 10% of other sugar units, providing that the sum of the components in the crude exopolysaccharide is 100 wt%.

4. The exopolysaccharide-protein complex according to any of claims 1-3, wherein the crude exopolysaccharide further comprises sulfate at a concentration from 2 to 10 wt %.

5. The exopolysaccharide-protein complex according to any of claims 1-4, wherein the exopolysaccharide-associated proteins include two bands of molecular weight corresponding to a band between 30 kDa and 40 kDa and a band between 51 kDa and 60 kDa as obtained by SDS-PAGE.

6. A method of preparing an exopolysaccharide-protein complex according to any of claims 1 to 5, comprising the steps of:

   - culturing one or more bacteria from genus selected from *Halomonas, Pseudoalteromonas, Vibrio, Salinivibrio, Marinomonas, Alteromonas, Pseudomonas, Halobacillus, Bacillus;*
   - isolating the exopolysaccharide-protein complex from the secreted fraction of the culture.

7. The method according to claim 6, wherein said bacteria genus is *Halomonas.*

8. The method according to claim 7, wherein the *Halomonas elongata sp.* is used as a bacterium belonging to Halomonas genus.

9. A composition comprising the exopolysaccharide-protein complex according to any of claims 1-5.

10. The composition according to claim 9, further comprising a biological response modifier selected from the group consisting of lymphokine, interleukin, growth factor and NFkB factor.

11. The composition according to claims 9 or 10 which is a pharmaceutical, nutraceutical or cosmeceutical composition.

12. The composition according to any of claims 9 to 11 for use in stimulating an immune response in a subject for preventing or treating a disease selected from cancer or a disease associated to undesirable inflammatory activity.

13. The composition for use according to claim 12, wherein said disease associated to undesirable inflammatory activity is selected from allergy, Alzheimer's disease, arthritis, autoimmune deficiency syndrome, celiac disease, diabetes mellitus, gastrointestinal disorder, inflammatory bowel disease, interstitial cystitis, skin disorders, acne, arteritis, arthritis, cancer, cellulitis, dermatitis and cardiovascular diseases.

14. The composition for use according to claim 12, wherein said cancer is liver cancer or a hepatocellular carcinoma.

15. The exopolysaccharide-protein complex according to any of claims 1-5 for use as an anticancer agent or adjuvant agent for cancer therapies.

Figure 1

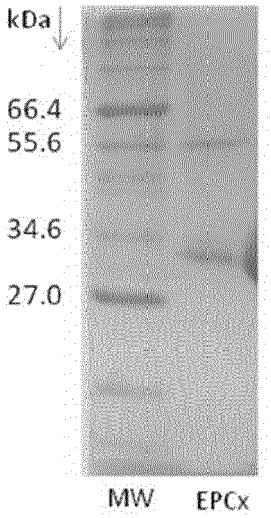

Figure 2

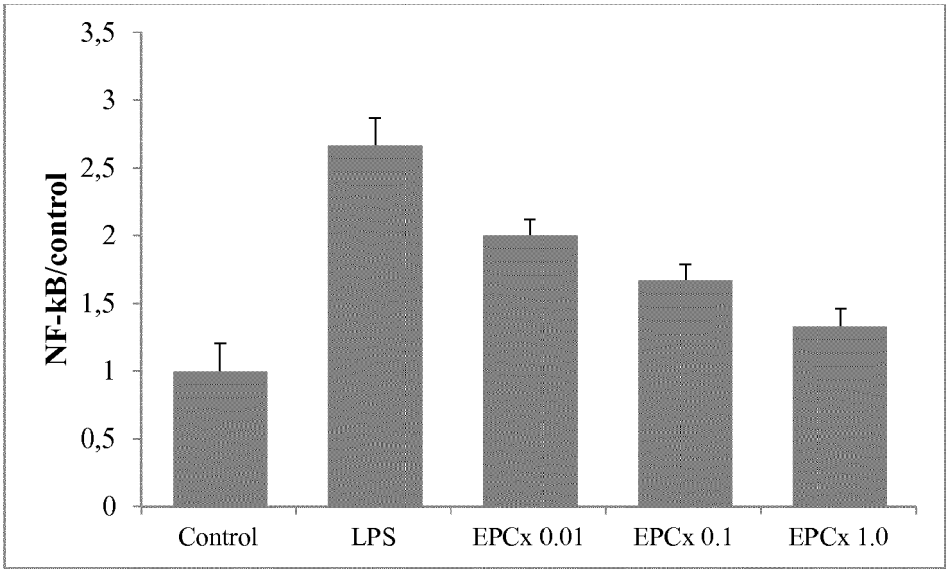
NF-kB/control
3,5
3
2,5
2
1,5
1
0,5
0
Control
LPS
EPCx 0.01
EPCx 0.1
EPCx 1.0

Figure 3.

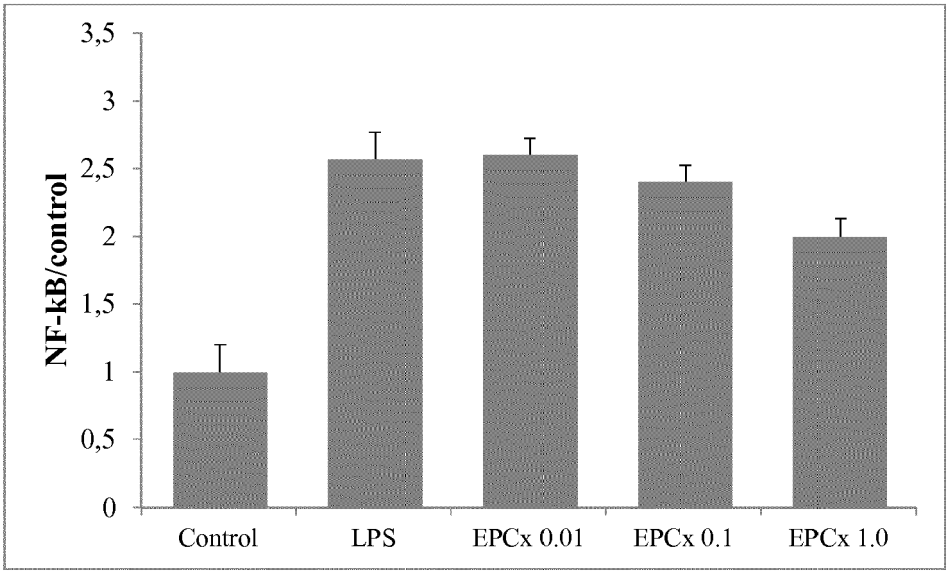
NF-kB/control
3,5
3
2,5
2
1,5
1
0,5
0
Control
LPS
EPCx 0.01
EPCx 0.1
EPCx 1.0

Figure 4

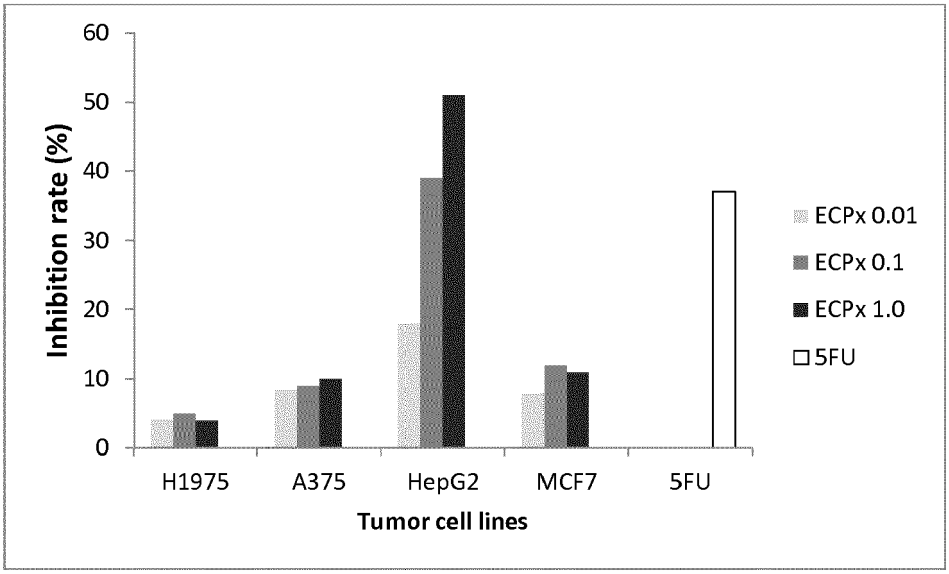
60
50
40
30
20
10
0
Inhibition rate (%)
H1975
A375
HepG2
MCF7
5FU
Tumor cell lines
ECPx 0.01
ECPx 0.1
ECPx 1.0
5FU

Figure 5

SEQ ID NO 1.

AACGATCCTAGCTTGCTAGGAGGCGTCGAGCGGCGGACGGGTGAGTAATGCATAGG
AATCTGCCCGGTAGTGGGGGATAACTTGAGGAAACTCAAGCTAATACCGCATACGCC
CTACGGGGGAAAGCAGGGGMTCTTCGGACCTTGCGCTATCGGATGAGCTTATGTCG
GATTAGCTGGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGA
GAGGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTG
AAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAATGCTTGTCGGTTAAT
ACCCGGCAAGGGAGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA
GCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTG
CGTAGGCGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCA
TCCGGAACTGTTAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGG
TGAAATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGA
CACTGACGCTGAGGTACGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCGACTAGCCGTTGGGGTCCTCGAGACCTTTGTGG

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 16 38 2436

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	FILOMENA FREITAS ET AL: "Advances in bacterial exopolysaccharides: from production to biotechnological applications", TRENDS IN BIOTECHNOLOGY, vol. 29, no. 8, 1 August 2011 (2011-08-01), pages 388-398, XP028241523, ISSN: 0167-7799, DOI: 10.1016/J.TIBTECH.2011.03.008 [retrieved on 2011-04-05]	1,2,9, 11,12,15	INV. A61K8/73
Y	* the whole document *	3,5,8,14	
X	ANTHONY COURTOIS ET AL: "Exopolysaccharides Isolated from Hydrothermal Vent Bacteria Can Modulate the Complement System", PLOS ONE, vol. 9, no. 4, 15 April 2014 (2014-04-15), page e94965, XP55253215, DOI: 10.1371/journal.pone.0094965	1,4,6,9, 11,12,15	
Y	* the whole document *	3,5,8,14	TECHNICAL FIELDS SEARCHED (IPC)
X	WO 2016/067218 A1 (LUBRIZOL ADVANCED MAT INC [US]) 6 May 2016 (2016-05-06)	1,2,6,7, 9-13	A61K
Y	* the whole document *	3,5,8,14	
X	US 2015/079137 A1 (DELGADO-GONZÁLEZ RAQUEL [ES] ET AL) 19 March 2015 (2015-03-19) * the whole document *	1,6,9-11	
X	WO 2015/063240 A1 (LIPOTEC S A U [ES]) 7 May 2015 (2015-05-07) * the whole document *	1,6,7,9, 11-13	
X	CN 104 694 594 A (UNIV DALIAN OCEAN; ZHANG FUYUN) 10 June 2015 (2015-06-10) * the whole document *	1,6,9, 11-13,15	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	8 February 2017	Luo, Xinmei

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 38 2436

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08-02-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016067218 A1	06-05-2016	NONE	
US 2015079137 A1	19-03-2015	AU 2013237387 A1	02-10-2014
		EP 2827837 A2	28-01-2015
		ES 2424294 A1	30-09-2013
		JP 2015510919 A	13-04-2015
		KR 20150005928 A	15-01-2015
		US 2015079137 A1	19-03-2015
		WO 2013139965 A2	26-09-2013
WO 2015063240 A1	07-05-2015	CN 105934231 A	07-09-2016
		EP 2868308 A1	06-05-2015
		EP 3062759 A1	07-09-2016
		JP 2016535035 A	10-11-2016
		KR 20160078458 A	04-07-2016
		US 2016263014 A1	15-09-2016
		WO 2015063240 A1	07-05-2015
CN 104694594 A	10-06-2015	NONE	

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2015063240 A1 **[0009]**
- WO 2015117985 A **[0010]**
- WO 2010023178 A1 **[0011]**
- US 7348420 B2 **[0012]**
- US 8129518 B2 **[0013]**
- US 8088605 B2 **[0014]**
- WO 2009127057 A1 **[0015]**
- CN 104694594 **[0016]**
- US 9095733 B **[0017]**
- WO 2013082915 A1 **[0018]**
- WO 2015063240A1 A, GarcÍA Sanz, M. N., Ferrer Montiel Antonio, V., Soley Astals, A., & AlmiÑAna DomÉNech, N. **[0088]**
- WO 2009127057A1 A, Loing, E., Briatte, S., Vayssier, C., Beaulieu, M., & Dionne, P. **[0088]**
- WO 2015117985A1 A, MercadÉ Gil Ma, E., CamON Fonseca, O., & Montes LOPez Ma, J. **[0088]**
- CN 104694594 A, Zhang, F., Liu, Y. E., Wang, W., Zhao, Y. U., & Li, Z. **[0088]**

Non-patent literature cited in the description

- **BAZANI CABRAL DE MELO, F. C. ; BORSATO, D. ; DE MACEDO JUNIOR, F. C. ; MANTOVANI, M. S. ; LUIZ, R. C. ; PEDRINE COLABONE CELLIGOI, M. A.** Study of levan productivity from Bacillus subtilis Natto by surface response methodology and its antitumor activity against HepG2 cells using metabolomic approach. *Pakistan Journal of Pharmaceutical Sciences,* 2015, vol. 28 (6), 1917-1926 **[0088]**
- **BEUTLER, B.** Innate immunity: an overview. *Molecular Immunology,* 2004, vol. 40 (12), 845-859 **[0088]**
- **CISZEK-LENDA, M. ; NOWAK, B. ; SROTTEK, M. ; GAMIAN, A. ; MARCINKIEWICZ, J.** Immunoregulatory potential of exopolysaccharide from Lactobacillus rhamnosus KL37. Effects on the production of inflammatory mediators by mouse macrophages. *International Journal of Experimental Pathology,* 2011, vol. 92 (6), 382-391 **[0088]**
- **DE VUYST, L. ; DE VIN, F. ; VANINGELGEM, F. ; DEGEEST, B.** Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *International Dairy Journal,* 2001, vol. 11 (9), 687-707 **[0088]**
- **FREITAS, F. ; ALVES, V. D. ; REIS, M. A. M.** Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends in Biotechnology,* 2011, vol. 29 (8), 388-398 **[0088]**
- **GAMAL-ELDEEN, A. M. ; AHMED, E. F. ; ABO-ZEID, M. A.** In vitro cancer chemopreventive properties of polysaccharide extract from the brown alga, Sargassum latifolium. *Food and Chemical Toxicology,* 2009, vol. 47 (6), 1378-1384 **[0088]**
- **GORSKA, S. ; SCHWARZER, M. ; JACHYMEK, W. ; SRUTKOVA, D. ; BRZOZOWSKA, E. ; KOZAKOVA, H. et al.** Distinct Immunomodulation of Bone Marrow-Derived Dendritic Cell Responses to Lactobacillus plantarum WCFS1 by Two Different Polysaccharides Isolated from Lactobacillus rhamnosus LOCK 0900. *Applied and Environmental Microbiology,* 2014, vol. 80 (20), 6506-6516 **[0088]**
- **HONDA, S. ; AKAO, E. ; SUZUKI, S. ; OKUDA, M. ; KAKEHI, K. ; NAKAMURA, J.** HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHY OF REDUCING CARBOHYDRATES AS STRONGLY ULTRAVIOLET-ABSORBING AND ELECTROCHEMICALLY SENSITIVE 1-PHENYL-3-METHYL-5-PYRAZOLONE DERIVATIVES. *Analytical Biochemistry,* 1989, vol. 180 (2), 351-357 **[0088]**
- **KIM, H. S. ; HONG, J. T. ; KIM, Y. ; HAN, S.-B.** Stimulatory Effect of beta-glucans on Immune Cells. *Immune network,* 2011, vol. 11 (4), 191-195 **[0088]**
- **LAEMMLI, U. K.** CLEAVAGE OF STRUCTURAL PROTEINS DURING ASSEMBLY OF HEAD OF BACTERIOPHAGE-T4. *Nature,* 1970, vol. 227 (5259), 680 **[0088]**
- **LI, J. ; QIAN, W. ; XU, Y. ; CHEN, G. ; WANG, G. ; NIE, S. et al.** Activation of RAW 264.7 cells by a polysaccharide isolated from Antarctic bacterium Pseudoaltermonas sp S-5. *Carbohydrate Polymers,* 2015, vol. 130, 97-103 **[0088]**
- **MIRANDA, C. C. B. O. ; DEKKER, R. F. H. ; SERPELONI, J. A. ; FONSECA, E. A. L. ; COULS, L. M. S. ; BARBOSA, A. M.** Anticlastogenic activity exhibited by botryosphaeran, a new exopolysaccharide produced by Botryosphaeria rhodina MAMB-05. *International Journal of Biological Macromolecules,* 2008, vol. 42 ((2)), 172-177 **[0088]**

- **MORAINE, R. A. ; ROGOVIN, P.** KINETICS OF POLYSACCHARIDE B-1459 FERMENTATION. *Biotechnology and Bioengineering,* 1966, vol. 8 (4), 511 **[0088]**
- **NEVOT, M. ; DERONCELE, V. ; LOPEZ-IGLESIAS, C. ; BOZAL, N. ; GUINEA, J. ; MERCADE, E.** Ultrastructural analysis of the extracellular matter secreted by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3. *Microbial Ecology,* 2006, vol. 51 (4), 501-507 **[0088]**
- **NEVOT, M. ; DERONCELE, V. ; MESSNER, P ; GUINEA, J. ; MERCADE, E.** Characterization of outer membrane vesicles released by the psychrotolerant bacterium Pseudoalteromonas antarctica NF3. *Environmental Microbiology,* 2006, vol. 8 (9), 1523-1533 **[0088]**
- **NWODO, U. U. ; GREEN, E. ; OKOH, A. I.** Bacterial Exopolysaccharides: Functionality and Prospects. *International Journal of Molecular Sciences,* 2012, vol. 13 (11), 14002-14015 **[0088]**
- **POLI, A. ; ANZELMO, G. ; NICOLAUS, B.** Bacterial Exopolysaccharides from Extreme Marine Habitats: Production, Characterization and Biological Activities. *Marine Drugs,* 2010, vol. 8 (6), 1779-1802 **[0088]**
- **QUESADA, E. ; BEJAR, V. ; CALVO, C.** EXOPOLYSACCHARIDE PRODUCTION BY VOLCANIELLA EURIHALINA. *Experientia,* 1993, vol. 49 (12), 1037-1041 **[0088]**
- **RAAFAT, E. M. ; GAMAL-ELDEEN, A. M. ; EL-HUSSIENY, E. A. ; AHMED, E. F. ; EISSA, A. A.** Polysaccharide extracts of the brown alga Sargassum asperifolium possess in vitro cancer chemopreventive properties. *Natural Product Research,* 2014, vol. 28 (24), 2304-2311 **[0088]**
- **RAINEY, F. A. ; WARDRAINEY, N. ; KROPPENSTEDT, R. M. ; STACKEBRANDT, E.** The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage. *Proposal of Nocardiopsaceae fam nov. International Journal of Systematic Bacteriology,* 1996, vol. 46 (4), 1088-1092 **[0088]**
- **RASULOV, M. M. ; KUZNETSOV, I. G. ; SLUTSKII, L. I. ; VELIKAIA, M. V. ; ZABOZLAEV, A. G ; VORONKOV, M. G.** The ulcerostatic effect of the exopolysaccharide from Bacillus mucilaginosus and its possible mechanisms. *Biulleten' eksperimental'noi biologii i meditsiny,* 1993, vol. 116 (11), 504-505 **[0088]**
- **SHAO, L. ; WU, Z. ; ZHANG, H. ; CHEN, W. ; AI, L. ; GUO, B.** Partial characterization and immunostimulatory activity of exopolysaccharides from Lactobacillus rhamnosus KF5. *Carbohydrate Polymers,* 2014, vol. 107, 51-56 **[0088]**
- **SUN, Z. ; LI, M. J. ; QI, Q. S. ; GAO, C. J. ; LIN, C. S. K.** Mixed Food Waste as Renewable Feedstock in Succinic Acid Fermentation. *Applied Biochemistry and Biotechnology,* 2014, vol. 174 (5), 1822-1833 **[0088]**
- **VENTOSA, A. ; GARCIA, M. T. ; KAMEKURA, M. ; ONISHI, H. ; RUIZBERRAQUERO, F.** BACILLUS-HALOPHILUS SP-NOV, A MODERATELY HALOPHILIC BACILLUS SPECIES. *Systematic and Applied Microbiology,* 1989, vol. 12 (2), 162-166 **[0088]**
- **YANG, J. ; LI, X. ; XUE, Y. ; WANG, N. ; LIU, W.** Anti-hepatoma activity and mechanism of corn silk polysaccharides in H22 tumor-bearing mice. *International Journal of Biological Macromolecules,* 2014, vol. 64, 276-280 **[0088]**
- **YANG, X. B. ; ZHAO, Y. ; WANG, Q. W. ; WANG, H. F. ; MEI, Q. B.** Analysis of the monosaccharide components in Angelica polysaccharides by high performance liquid chromatography. *Analytical Sciences,* 2005, vol. 21 (10), 1177-1180 **[0088]**
- **YANG, X. B. ; ZHAO, Y. ; ZHOU, S. Y. ; LIU, L. ; WANG, H. F. ; MEI, Q. B.** Analysis of monosaccharide composition in Angelica polysaccharides by pre-column derivatization high performance liquid chromatography. *Chinese Journal ofAnalytical Chemistry,* 2005, vol. 33 (9), 1287-1290 **[0088]**
- **YASUDA, E. ; SERATA, M. ; SAKO, T.** Suppressive Effect on Activation of Macrophages by Lactobacillus casei Strain Shirota Genes Determining the Synthesis of Cell Wall-Associated Polysaccharides. *Applied and Environmental Microbiology,* 2008, vol. 74, 4746 **[0088]**
- *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* vol. 75 (4), 1221-1221 **[0088]**
- **YE, S. ; ZHANG, J. ; LIU, Z. ; ZHANG, Y. ; LI, J. ; LI, Y. O.** Biosynthesis of selenium rich exopolysaccharide (Se-EPS) by Pseudomonas PT-8 and characterization of its antioxidant activities. *Carbohydrate Polymers,* 2016, vol. 142, 230-239 **[0088]**
- **ZHENG, Y. ; WANG, W.-D. ; LI, Y.** Antitumor and immunomodulatory activity of polysaccharide isolated from Trametes orientalis. *Carbohydrate Polymers,* 2015, vol. 131, 248-254 **[0088]**